ION CHANNEL

Voltage-gated sodium channels are transmembrane proteins which cause sodium permeability to increase. Depolarization of the plasma membrane causes sodium channels to open allowing sodium ions to enter along the electrochemical gradient creating an action potential.

Voltage-gated sodium channels are expressed by all electrically excitable cells, where they play an essential role in action potential propagation. They comprise a major subunit of about 2000 amino acids which is divided into four domains (D1-D4), each of which contains 6 membrane-spanning regions (S1-S6). The alpha-subunit is usually associated with 2 smaller subunits (beta-1 and beta-2) that influence the gating kinetics of the channel. These channels show remarkable ion selectivity, with little permeability to other monovalent or divalent cations. Patch-clamp studies have shown that depolarisation leads to activation with a typical conductance of about 20pS, reflecting ion movement at the rate of 10^7 ions/second/channel. The channel inactivates within milliseconds (Caterall, W.A., Physiol. Rev. 72, S4-S47 (1992); Omri et al, J. Membrane Biol 115, 13-29; Hille, B, Ionic Channels in Excitable Membranes, Sinauer, Sunderland, MA (1991)).

Sodium channels have been pharmacologically characterised using toxins which bind to distinct sites on sodium channels. The heterocyclic guanidine-based channel blockers tetrodotoxin (TTX) and saxitoxin (STX) bind to a site in the S5-S6 loop, whilst μ-conotoxin binds to an adjacent overlapping region. A number of toxins from sea anemones or scorpions binding at other sites alter the voltage-dependence of activation or inactivation.

Voltage-gated sodium channels that are blocked by nanomolar concentrations of tetrodotoxin are known as tetrodotoxin sensitive sodium channels (Hille (1991) "Ionic Channels in Excitable Membranes", Sinauer Sunderland, MA (1991)) whilst sodium channels that are blocked by concentrations greater than 1 micromolar are known as tetrodotoxin-insensitive (TTXi) sodium channels (Pearce and Duchen Neuroscience 63, 1041-1056 (1994)).

Dorsal root ganglion (DRG) neurons express at least three types of sodium channels which differ in kinetics and sensitivity to TTX. Neurons with small-diameter cell bodies and unmyelinated axons (C-fibers) include most of the nociceptor (damage-sensing)

population and express a fast TTX-sensitive current and a slower TTX-insensitive current. Of the five cloned sodium channel α-subunit transcripts known to be present in dorsal root ganglia, none exhibits the properties of the TTX-insensitive channel.

Sodium channel blockers are used clinically to provide pain relief. Three classes of sodium channel blockers in common clinical use are: local anesthetics such as lidocaine, some anticonvulsants such as phenytoin and carbamazepine, and some antiarrhythmics such as mexiletine. Each of these is known to suppress ectopic peripheral nervous system discharge in experimental preparations and to provide relief in a broad range of clinical neuropathic conditions.

Applicants have now found a novel voltage-gated sodium channel (hereinafter referred to as a sodium channel specifically located in sensory neurons or also referred to as SNS sodium channel) that is present in sensory neurons (or neurones) but not present in glia, muscle, or the neurons of the sympathetic, parasympathetic, enteric or central nervous systems. Preferably the sodium channel of the invention is found in the neurons of the dorsal root ganglia (DRG) or cranial ganglia. More preferably the sodium channel of the invention is found in the neurons of the dorsal root ganglia. Preferably the sodium channel is specifically located in rat sensory neurons or human sensory neurons.

The sodium channel of the present invention is believed to play a role in nociceptive transmission because some noxious input to the central nervous system is known to be insensitive to TTX. Persistent activation of peripheral nociceptors has been found to result in changes in excitability in the dorsal horn associated with the establishment of chronic pain. Increased sodium channel activity has also been shown to underlie neuroma-induced spontaneous action potential generation. Conversely, chronic pain may be successfully treated by surgical or pharmacological procedures which block peripheral nerve activation. Blockage of nociceptor input may therefore produce useful therapeutic effects, even though central nervous system plasticity plays a pivotal role in the establishment of chronic pain. Sensory neuron-specific voltage-gated sodium channels, particularly sub-types associated with a nociceptive modality such as the sodium channel of the invention, thus provide targets for therapeutic intervention in a range of pain states. The electrophysiological and pharmacological properties of the expressed SNS sodium channel are similar to those described for the small diameter sensory neuron tetrodotoxin-resistant sodium channels. As some noxious input into the spinal cord is resistant to

tetrodotoxin, block of expression or function of such a C-fiber-restricted sodium channel may have a selective analgesic effect.

In another aspect the present invention provides an isolated protein comprising a sodium channel specifically located in rat sensory neurons as encoded by the insert deposited in NCIMB deposit number 40744, which was deposited at The National Collections of Industrial and Marine Bacteria, 23 St Machar Drive, Aberdeen AB2 1RY, Scotland, United Kingdom on 27 June 1995 in accordance with the Budapest Treaty.

The invention also provides nucleotide sequences coding for the SNS sodium channel. In a preferred embodiment, the nucleotide sequence encodes a sodium channel specifically located in rat sensory neurons which is as set out in Figure 1a or a complementary strand thereof.

The approximately 6.5 kilobase (kb) transcript expressed selectively in rat dorsal root ganglia that codes for the novel sodium channel of the invention shows sequence similarities with known voltage-gated sodium channels. The cDNA codes for a 1,957 amino acid protein. In particular, the novel sodium channel of the invention shows 65% identity at the amino acid level with the rat cardiac tetrodotoxin-insensitive (TTXi) sodium channel. The aromatic residue that is involved in high-affinity binding of TTX to the channel atrium of TTX-sensitive sodium channels is altered to a hydrophilic serine in the predicted protein of the SNS sodium channel, whereas the residues implicated in sodium-selective permeability are conserved. The novel sodium channel specifically located in sensory neurons shows relative insensitivity to TTX (IC50>1 micromolar) and thus exhibits properties different from other cloned sodium channel transcripts known to be present in dorsal root ganglia.

The invention also provides expression and cloning vectors comprising a nucleotide sequence as hereinabove defined. In order to effect transformation, DNA sequences containing the desired coding sequence and control sequences in operable linkage (so that hosts transformed with these sequences are capable of producing the encoded proteins) may be included in a vector, however, the relevant DNA may then also be integrated into the host chromosome.

The invention also provides a screening assay for modulators of the sodium channel which is specifically located in sensory neurons wherein the assay comprises

adding a potential modulator to a cell expressing the SNS sodium channel and detecting any change in activity of the sodium channel.

The present invention also provides a modulator which has activity in the screening assay hereinabove defined. Modulators of the sodium channel as hereinabove defined are useful in modulating the sensation of pain. Blockers of the sodium channel will block or prevent the trasmission of impulses along sensory neurons and thereby be useful in the treatment of acute, chronic or neuropathic pain.

The present invention thus relates to novel voltage-gated sodium channel proteins specific to sensory neurons, to nucleotide sequences capable of encoding these sodium channel proteins, to vectors comprising a nucleotide sequence coding for a sodium channel of the invention, to host cells containing these vectors, to cells transformed with a nucleic acid sequence coding for the sodium channel, to screening assays using the sodium channel proteins and/or host cells, to complementary stands of the DNA sequence which is capable of encoding the sodium channel proteins and to antibodies specific for the sodium channel proteins. These and other aspects of the present invention are set forth in the following detailed description.

Brief Description of the Drawings:

~~**Figure 1a shows the nucleic acid and amino acid sequences of the sodium channel specific to the rat DRG (SNS-B) (SEQ ID NO: 1 and SEQ ID NO: 2).**~~

Figure 1b shows the structure of the SNS-B voltage-gated sodium channel in pGEM-3Z.

Figure 1c shows a schematised drawing of a known voltage-gated sodium channel.

Figure 2 shows sequences of examples of PCR primers for isolation of human clone probes. RLLRVFKLAKSWPTL - SEQ ID NO: 21; 5' gcttgctgcgggtcttcaagc 3' SEQ ID NO: 22; LRALPLRALSRFEG - SEQ ID NO: 23; 5' atcgagacagagcccgcagcg 3' SEQ ID NO: 24; 5' acgggtgccgcaaggacggcgtctccgtgtggaacggcgagaag 3' SEQ ID NO: 25; and 5' ggctatccttcctcttccagctctcacccaggtatggagccaggt 3' - SEQ ID NO: 26.

Figure 3 shows a film of ^{35}S radio-labelled SNS-B voltage-gated sodium channel protein in a coupled transcription/translation system.

Figure 4a and **Figure 4b** show SNS-GST fusion protein constructs for antibody generation. TCCCGTACGCTGCAGCTCTTT - SEQ ID NO: 27; CCCGGGGAAGGCTAC - SEQ ID NO: 28; GTCGACACCAGAAAT - SEQ ID NO: 29; GGATCCTCTAGAGTCGACCTGCAGAAGGAA - SEQ ID NO: 30

In accordance with one aspect of the invention there is provided an isolated and/or purified nucleic acid sequence (or polynucleotide or nucleotide sequence) which comprises a nucleic acid sequence which encodes the mammalian sodium channel specifically located in sensory neurons or a complementary strand thereof. Preferably, the nucleic acid sequence encodes the sodium channel specifically located in mammalian dorsal root ganglia. More preferably, the nucleic acid sequence encodes the rat or human sodium channel specifically located in dorsal root ganglia. The rat nucleic acid sequence preferably comprises the sequence of the coding portion of the nucleic acid sequence shown in Figure 1a (SEQ ID NO:1) or the coding portion of the cDNA deposited in NCIMB deposit number 40744 which was deposited at the National Collections of Industrial and Marine Bacteria, 23 St. Machar Drive, Aberdeen AB21RY, Scotland, United Kingdom on June 27, 1995 in accordance with the Budapest Treaty.

A nucleic acid sequence encoding a sodium channel of the present invention may be obtained from a cDNA libraray derived from mammalian sensory neurons, preferably dorsal root ganglia, trigeminal ganglia or other cranial ganglia, more preferably rat or human dorsal root ganglia. The nucleotide sequence described herein was isolated from a cDNA library derived from rat dorsal root ganglia cells. The nucleic acid sequence coding for the SNS sodium channel has an open reading frame of 5,871 nucleotides encoding a 1,957 amino acid protein. A nucleic acid sequence encoding a sodium channel of the present invention may also be obtained from a mammalian genomic library, preferably a human or rat genomic library. The nucleic acid sequence may be isolated by the subtraction hybridization method described in the examples, by screening with a probe derived from the rat sodium channel sequence, or by other methodologies known in the art such as polymerase chain reaction (PCR) with appropriate primers derived from the rat sodium channel sequence and/or relatively conserved regions of known voltage-gated sodium channels.

The nucleic acid sequences of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic

DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the rat SNS sodium channel or variant thereof may be identical to the coding sequences set forth herein or that of the deposited clone, or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same protein as the sequences set forth herein or the deposited cDNA.

The nucleic acid sequence which encodes the SNS sodium channel may include: only the coding sequence for the full length protein or any variant thereof; the coding sequence for the full length protein or any variant thereof and additional coding sequence such as a leader or secretory sequence or a proprotein sequence; the coding sequence for the full length protein or any variant thereof (and optionally additional coding sequence) and non-coding sequences, such as introns or non-coding sequences 5' and/or 3' of the coding sequence for the full length protein.

The present invention further relates to variants of the hereinabove described nucleic acid sequences which encode fragments, analogs, derivatives or splice variants of the SNS sodium channel. The variant of the SNS sodium channel may be a naturally occurring allelic variant of the SNS sodium channel. As known in the art, an allelic variant is an alternate form of a protein sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded protein. The present invention relates to splice variants of the SNS sodium channel that occur physiologically and which may play a role in changing the activation threshold of the sodium channel.

Variants of the sequence coding for the rat SNS sodium channel have been identified and are listed below:

1) a 2573 base pair nucleic acid sequence shown in SEQ ID NO:3. This sequence codes for a 521 amino acid protein that corresponds to amino acids 1437-1957 of Figure 1a (SEQ ID NO:1) and has the same sequence as bases 4512 through 6524 of Figure 1a in the coding portion and 3' untranslated region.

2) a 7052 base pair nucleic acid sequence shown in SEQ ID NO: 5. SEQ ID NO: 5 codes for a 2,132 amino acid protein that contains a 176 amino acid repeat (amino acids 586-760 of SEQ ID NO:6) inserted after amino acid 585 in Figure 1a or SEQ ID NO:2.

A preferred sequence for the rat SNS sodium channel is shown in Figure 1a (SEQ ID NO: 1). However, sequencing variations have been noted. Sequencing has provided

a 6,321 base pair nucleic acid sequence coding for a 1957 amino acid protein that has the same base sequence as bases 1-6321 of Figure 1a or SEQ ID NO:1 with the following changes: bases 1092 G to A, base 1096 C to T, base 2986 G to T, base 3525 C to G and base 3556 G to C.

a 6,527 base pair nucleic acid sequence coding for a 1,957 amino acid protein as shown in SEQ ID NO:7 that has the same base sequence as bases 1-6524 of Figure 1a (SEQ ID NO:1) with an additional 3 bases AAA, at the 3' end, and the following changes: base 299 C to G, base 1092 G to A, base 1096 C to T, base 1964 G to C, base 1965 C to G, base 2472 A to T, base 2986 G to T, base 3019 A to G, base 3158 C to T, base 3525 C to G, base 3556 G to C and base 5893 T to G. The sequence of SEQ ID NO: 7 is also a preferred sequence coding for the rat SNS sodium channel.

a 6524 base pair nucleic acid sequence that has the same sequence as Figure 1a (SEQ ID NO: 1) except for the following base changes: base 1092 G to A (resulting in a change at amino acid 297 of SEQ ID NO: 2 from Val to Ile), base 1096 C to T (resulting in a change at amino acid 298 from Ser to Phe), base 1498 C to A (resulting in a change at amino acid 432 from Ala to Glu), and base 2986 G to T (resulting in a change at amino acid 928 form Ser to Ile).

Sequence variability has been identified in different isolates. One such seqeuence has been identified that has the sequence of the third sequencing variation shown immediately above except for eight base differences, five of which resulted in an altered amino acid sequence F16-S16, L393-P393, T470-I470, R278-H278, and I1,876-M1,876.

The present invention also relates to nucleic acid probes constructed from the nucleic acid sequences of the invention or portion thereof. Such probes could be utilized to screen a dorsal root ganglia cDNA library to isolate a nucleic acid sequence encoding the sodium channel of the present invention. The nucleic acid probes can include portions of the nucleic acid sequence of the SNS sodium channel or variant thereof useful for hybridizing with mRNA or DNA for use in assays to detect expression of the SNS

sodium channel or localize its presence on a chromosome, such as the *in situ* hybridization assay described herein.

A conservative analogue is a protein sequence which retains substantially the same biological properties of the sodium channel but differs in sequences by one or more conservative amino acid substitutions. For the purposes of this document a conservative amino acid substitution is a substitution whose probability of occuring in nature is greater than ten times the probability of that substitution occuring by chance (as defined by the computational methods described byDayhoff et al, Atlas of Proteins Sequence and Structure, 1971, page 95-96 and figure 9-10).

A splice variant is a protein product of the same gene, generated by alternative splicing of mRNA, that contains additions or deletions within the coding region (Lewin B. (1995) Genes V Oxford University Press, Oxford, England)

The nucleic acid sequences of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the protein of the present invention such as a hexa-histidine tag or a hemagglutinin (HA) tag.

The present invention further relates to nucleic acid sequences which hybridize to the hereinabove-described sequences if there is at least 50% and preferably 70% identity between the sequences. The present invention particularly relates to nucleic acid sequences which hybridize under stringent conditions to the hereinabove-described nucleic acid sequences. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences preferably the nucleic acid sequences which hybridize to the hereinabove described nucleic acid sequences encode proteins which retain substantially the same biological function or activity as the SNS sodium channel, however, nucleic acid sequences that have different properties are also within the scope of the present invention. Such sequences, while hybridizing with the above described nucleic acid sequences may encode a protein having diffferent properties, such as sensitivity to tetrodotoxin which property is found in the altered SNS sodium channel protein described herein.

In accordance with another aspect of the invention there is provided purified mammalian sensory neuron sodium channel protein, wherein the sodium channel is insensitive to tetrodotoxin. Preferably the sodium channel of the invention is found in the neurons of the dorsal root ganglia or cranial ganglia, more preferably the neurons of the

dorsal root ganglia. The sodium channel protein may be derived from any mammalian species, preferably the rat or human sodium channel protein. The rat SNS sodium channel protein preferably has the deduced amino acid sequence shown in Figure 1a (SEQ ID NO:2) or SEQ ID NO: 8, or the amino acid sequence encoded by the deposited cDNA. Fragments, analogues, derivatives, and splice variants of the sodium channel specifically located in sensory neurons are also within the scope of the present invention.

The terms "fragment," "derivative" and "analogue" when referring to the DRG sodium channel of the invention refers to a protein which retains substantially the same biological function or activity as such protein. Thus, an analogue includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature protein. In addition, the present invention also includes derivatives wherein the biological function or activity of the protein is significantly altered, including derivatives that are sensitive to tetrodotoxin.

The protein of the present invention may be a recombinant protein, a natural protein or a synthetic protein, preferably a recombinant protein.

The fragment, derivative or analog of the SNS sodium channel protein includes, but is not limited to, (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituted group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the protein (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature protein, such as a leader or secretory sequence or a sequence which is employed for purification of the mature protein or a proprotein sequence, or (v) one in which one or more amino acids has/have been deleted so that the protein is shorter than the full length protein. Variants of the rat SNS sodium channel are discussed hereinabove and shown in SEQ ID NO:4 and SEQ ID NO:6.

The proteins and nucleic acid sequences of the present invention are preferably provided in an isolated form, and preferably are purified to at least 50% purity, more preferably about 75% purity, most preferably about 90% purity.

The terms "isolated" and/or "purified" mean that the material is removed from is original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring nucleic acid sequence or protein present in a living animal is not isolated or purified, but the same nucleic acid sequence or DNA or protein, separated from some or all of the coexisting materials in the natural system, is isolated or purified. Such nucleic acid sequence could be part of a vector and/or such nucleic acid sequence or protein could be part of a composition, and still be isolated or purified in that such vector or composition is not part of its natural environment.

The present invention also provides vectors comprising a nucleic acid sequence of the present invention, and host cells transformed or transfected with a nucleic of the invention.

The nucleic acid sequences of the present invention may be employed for producing the SNS sodium channel protein or variant thereof by recombinant techniques. Thus, for example, the nucleic acid sequence may be included in any one of a variety of expression vehicles or cloning vehicles, in particular vectors or plasmids for expressing a protein. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences. Examples of suitable vectors include derivatives of SV40; bacterial plasmids; phage DNA; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, pseudorabies and baculovirus. However, any other plasmid or vector may be used as long as it is replicable and viable in the host.

More particularly, the present invention also provides recombinant constructs comprising one or more of the nucleic acid sequences as broadly described above. The constructs comprise an expression vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises one or more regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen) pBs, phagescript, psiX174, pBluescript SK, pBsKS, pNH8a, pNH16a, pNH18a, pNH461 (Stratagene); pTrc99A, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLneo, pSV2cat,

pOG44, pXT1, pSG (Stratagene), pSVK3, pBPV, pMSG, pSVL (Pharmacia) pcDNA 3.1 (Invitrogen, San Diego, CA), pEE14 (WO 87/04462) and pREP8 (Invitrogen). Preferred vectors include pcDNA 3.1, pEE14 and pREP8. However, any other plasmid or vector may be used as long as it is replicable and viable in the host.

As hereinabove indicated, the appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into appropriate restriction endonuclease sites by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector may contain a ribosome binding site for translation initiation and transcription terminator. The vector may also include appropriate sequences for amplifying expression.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include LacI, LacZ, T3, T7, gpt, lambda P_R, P_L and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

Depending on the expression system employed in addition, the expression vectors preferably contain a gene to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli*.

Transcription of DNA encoding the protein of the present invention by higher eukaryotes can be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Examples include the SV40 enhancer on the late side of the replication origin (bp 100 to 270), a cytomegalovirus early promoter enhancer, a polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Useful expression vectors for bacterial use may be constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli, Bacillus subtilis, Salmonella typhimurium* and various species within the genera Pseydomonas, Streptomyces, and Staphylococcus, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, PKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, Wis., U.S.A.). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

The sodium channel can be expressed in insect cells with the baculovirus expression system which uses baculovirus such as Autographa Californica nuclear polyhydrosis virus (AcNPV) to produce large amounts of protein in insect cells such as the Sf9 or 21 clonal lines derived from *Spodoptera frugiperda* cells. See for example O'Reilly et al., (1992) Baculovirus Expression Vectors: A Laboratory Manual, Oxford University Press.

Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early promoter, enhancer, splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 viral

genome, for example, SV40 origin, early promoter, enhancer, splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

In a further embodiment, the present invention provides host cells capable of expressing a nucleic acid sequence of the invention. The host cell can be, for example, a higher eukaryotic cell, such as a mammalian cell, a lower eukaryotic cell, such as a yeast cell, a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell may be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, electroporation (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, 1986) or any other method known in the art.

Host cells are genetically engineered (transduced, transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the SNS sodium channel genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein. As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as *E. coli,* and *Salmonella typhimurium;* Streptomyces; fungal cells, such as yeast; insect cells such as Drosophila and *Spodoptera fugiperda* Sf9; animal cells such as CHO, COS or Bowes melanoma Ltk^- - and Y1 adrenal carcinoma; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art based on the teachings herein. Preferred host cells include mammalian cell lines such as CHO-K1, COS-7; Y1 adrenal; carcinoma cells. More preferably, the host cells are CHO-K1 cells. Preferred host cells for transient expresion of the SNS sodium channel include *Xenopus laevis* oocytes.

The sodium channel may be transiently expressed in *Xeropus laevis* oocytes. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and

expression vectors for use with prokaryotic and eukaryotic hosts are described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989).

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, CHO-K1, HeLa, HEK 293, NIH 3T3 and BHK cell lines.

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the proteins of the invention can be synthetically produced by conventional peptide synthesizers.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well-known to those skilled in the art.

The SNS sodium channel protein is recovered and purified from recombinant cell cultures by methods known in the art, including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, hydroxyapatite chromatography and lectin chromatography. Protein refolding steps may be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The SNS sodium channel protein of the present invention may be naturally purified products expressed from a high expressing cell line, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture).

The present invention also provides antibodies specific for the SNS sodium channel hereinabove defined. The term antibody as used herein includes all immunoglobulins and fragments thereof which contain recognition sites for antigenic

determinants of proteins of the present invention. The antibodies of the present invention may be polyclonal or preferably monoclonal, may be intact antibody molecules or fragments containing the active binding region of the antibody, e.g. Fab or $F(ab)_2$ and can be produced using techniques well established in the art [see e.g. R.A DeWeger et al; Immunological Rev., 62 p29-45 (1982)].

The proteins, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present also includes chimeric, single chain and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the SNS sodium channel can be obtained by direct injection of the polypeptide into an animal or by administering the protein to an animal, preferably a nonhuman. The antibody so obtained will then bind the protein itself. In this manner, even a sequence encoding only a fragment of the protein can be used to generate antibodies binding the whole native protein. Such antibodies can then be used to locate the protein in tissue expressing that polypeptide. For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, Nature 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, 35 al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss., pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention.

The antibodies of the present invention may also be of interest in purifying a protein of the present invention and accordingly there is provided a method of purifying a protein of the present invention as hereinabove defined or any portion thereof or a metabolite or degration product thereof which method comprises the use of an antibody of the present invention.

The purification method of the present invention may be effected by any convenient technique known in the art for example by providing the antibody on a support and contacting the antibody with a solution containing the protein whereby the antibody binds to the protein of the present invention. The protein may be released from binding with the antibody by known methods for example by changing the ionic strength of the solution in contact with the complex of the protein/antibody.

The present invention also provides methods of identifying modulators of the sodium channel which is specifically located in sensory neurons comprising contacting a test compound with the sodium channel and detecting the activity of the sodium channel. Preferably, the methods of identifying modulators or screening assays employ transformed host cells that express the sodium channel. Typically, such assays will detect changes in the activity of the sodium channel due to the test compound, thus identifying modulators of the sodium channel. Modulators of the sodium channel are useful in modulating the sensation of pain. Blockers of the sodium channel will prevent the transmission of impulses along sensory neurons and thereby be useful in the treatment of acute, chronic or neuropathic pain.

The sodium channel can be used in a patch clamp or other type of assay, such as the assays disclosed herein in the examples, to identify small molecules, antibodies, peptides, proteins, or other types of compounds that inhibit, block, or otherwise interact with the sodium channel. Such modulators identified by the screening assays can then be used for treatment of pain in mammals.

For example, host cells expressing the SNS sodium channel can be employed in ion flux assays such as $^{22}Na+$ ion flux and ^{14}C guanidinium ion assays, as described in the examples and in the art, as well as the SFBI fluorescent sodium indicator assays as described in Levi et al., (1994) J. Cardiovascular Electrophysiology 5:241-257. Host cells expressing the SNS sodium channel can also be employed in binding assays such as the 3H-batrachotoxin binding assay described in Sheldon et al., (1986) Molecular Pharmacology 30:617-623; the 3H-saxitoxin assay as described in Rogart et al (1983) Proc. Natl. Acad. Sci. USA 80:1106-1110; and the scorpion toxin assay described in West et al., (1992) Neuron 8:59-70. Additionally, the host cells expressing the SNS sodium channel can be used in electrophysiological assays using patch clamp or two electrode techniques. In general, a test compound is added to the assay and its effect on sodium flux is

determined or the test compound's ability to competitively bind to the sodium channel is assessed. Test compounds having the desired effect on the SNS sodium channel are then selected. Modulators so selected can then be used for treating pain as described above.

Complementary strands of the nucleotide sequences as hereinabove defined can be used in gene therapy, such as disclosed in U.S. Patent 5,399,346. For example, the cDNA sequence or fragments thereof could be used in gene therapy strategies to down regulate the sodium channel. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a nucleic acid sequence to DNA or RNA. For example, the 5' coding portion of the nucleic acid sequence that encodes the sodium channel is used to design an antisense RNA oligonucleotide of from about 10 to about 40 base pairs in length. A DNA oligonucleotide is designed to be complimentary to a region of the gene involved in transcription (triple helix - see Lee et al., Nucl. Acids Res. 6:3073 (1979); Cooney et al, Science 241:456 (1988); and Deruau et al., Science 251:1360 (1991)), thereby preventing transcription and the product of the sodium channel. The antisense RNA oligonucleotide hybridizes to the mRNA in vivo and blocks translation of the mRNA into the sodium channel. Antisense oligonucleotides or an antisense construct driven by a strong constituitive promoter expressed in the target sensory neurons would be delivered either peripherally or to the spinal cord.

The regulatory regions controlling expression of the sodium channel gene could be used in gene therapy to control expression of a therapeutic construct in cells expressing the sodium channel.

Such regions would be isolated by using the cDNA as a probe to identify genomic clones carrying the gene and also flanking sequence e.g. cosmids. Fragments of the cosmids containing intron or flanking sequence would be used in a reporter gene assay in e.g. DRG cultures or transgenic animals and genomic fragments carrying e.g. promoter, enhancer or LCR activity identified.

The invention will now be further described with reference to the following examples:

Example 1 - Derivation of the sequence of a rat dorsal root ganglia (DRG) sodium channel cDNA by subtraction hybridisation methodology

1.1 cDNA synthesis from DRG-derived poly-A+ RNA

Dorsal root ganglia (DRG) from all spinal levels of neonatal Sprague-Dawley male and female rats were frozen in liquid nitrogen. RNA is extracted using guanidine isothiocyanate and phenol/chloroform extraction (Chomczynski and Sacchi 1987 Anal Biochem 162,156-159).

Total RNA isolation - the nerve tissue is homogenised using a Polytron homogeniser in 1ml extraction buffer (23.6g guanidinium isothiocyanate, 5ml of 250 mM sodium citrate (pH 7.0) made up to 50ml with distilled water. To this is added 2.5ml 10% sarcosyl and 0.36ml ß-mercaptoethanol). 0.1ml of 2M sodium acetate (pH 4.0) is added followed by 1 ml phenol. After mixing, 0.2ml chloroform is added and this is shaken vigorously and placed on ice for 5 minutes. This is then centrifuged at 12,000 revolutions per minute (rpm) for 30 minutes at 4°C. The aqueous phase is transferred to a fresh tube, 1ml of isopropanol is added and this is left at -20°C for an hour followed by centrifuging at 12000 rpm for 30 minutes at 4°C. The pellet is dissolved in 0.1ml extraction buffer and is again extracted with isopropanol. The resulting pellet is washed with 70% ethanol and is resuspended in diethyl pyrocarbonate (DEPC)-treated water. 0.3M sodium acetate (pH5.2) and 2 volumes of ethanol are added and the mixture is placed at -20°C for 1 hour. The RNA is precipitated, washed again with 70% ethanol and resuspended in DEPC-treated water. The optical density is measured at 260 nanometres (nm) to calculate the yield of total RNA. Poly A+ RNA is isolated from the total RNA by oligo-dT cellulose chromatography (Aviv and Leder 1972 Proc Natl Acad Sci 69,1408-1411). The following procedures are carried out at 4°C as far as is possible. Oligo-dT cellulose (Sigma) is prepared by treatment with 0.1M sodium hydroxide for 5 minutes. The oligo-dT resin is poured into a column and is neutralised by washing with neutralising buffer (0.5 M potassium chloride, 0.01M Tris (Trizma base - Sigma - Tris(hydroxymethyl)aminomethane) (pH 7.5). The RNA solution is adjusted to 0.5M potassium chloride, 0.01M Tris (pH7.5) and is applied to the top of the column. The first column eluate is re-applied to the column to ensure sticking of the mRNA to the oligo-dT in the column. The column is then washed with 70ml of neutralising buffer and the polyA+ RNA is eluted with 6ml 0.01M Tris (pH7.5) and 1ml fractions are collected. The poly A+ RNA is usually in fractions 2 to 5 and this is checked by measuring the optical density at

260nm. These fractions are pooled and ethanol precipitated overnight at -70°C, washed in 70% ethanol and then redissolved in deionised water at a concentration of 1mg/ml.

First strand cDNA was generated using 0.5mg DRG poly A+ mRNA, oligo-dT/Not-I primer adapters and SuperScript reverse transcriptase (Gibco-BRL) using methodology as described in example 2. One half of the cDNA was labelled by including 2 MBq ^{32}P dCTP (Amersham) in the reverse transcriptase reaction. Labelled cDNA is separated from unincorporated nucleotides on Nick columns (Sephadex G50 - Pharmacia).

1.2 Enrichment of DRG-specific cDNA using subtraction hybridisation.

Poly A+ RNA from various tissues (10µg) is incubated with 10µg photoactivatable biotin (Clontech) in a total volume of 15µl and irradiated at 4°C for 30 minutes with a 250 watt sunlamp. The photobiotin is removed by extraction with butanol, and the cDNA co-precipitated with the biotinylated RNA without carrier RNA (Sive and St. John 1988 Nuc Ac Res 16,10937).

Hybridisation is carried out at 58°C for 40 hours in 20% formamide, 50mM 3-(N-morpholino)propanesulphonic acid (MOPS) (pH 7.6), 0.2% sodium dodecyl sulphate (SDS), 0.5M sodium chloride, 5mM ethylenediaminetetraacetate (EDTA - Sigma). The total reaction volume is 5µl and the reaction is carried out under mineral oil, after an initial denaturation step of 2 minutes at 95°C. 100µl 50mM MOPS (pH 7.4), 0.5M sodium chloride, 5mM EDTA containing 20 units of streptavidin (BRL) is then added to the reaction mixture at room temperature, and the aqueous phase retained after two phenol /chloroform extraction steps. After sequential hybridisation of the cDNA from Example 1.1 with biotinylated mRNA from liver and kidney, followed by cortex and cerebellum, a 80-fold concentration of DRG-specific transcripts is achieved.

One third of the 1-2 ng of residual cDNA is then G-tailed with terminal deoxynucleotide transferase at 37°C for 30 minutes. The polymerase chain reaction is used to amplify the cDNA using an oligo-dT-Not-I primer adapter and oligo-dC primers starting with the sequence AATTCCGA$(C)_{10}$. Amplification is carried out using 2 cycles of 95°C for 1min, 45°C for 1 min, 72°C for 5min, followed by 2 cycles of 95°C for 1 minute , 58°C for 1 minute and 72°C for 5 minutes. The resulting products are then separated on a

2% Nu-sieve agarose gel, and material running at a size of greater than 0.5 kilobase pairs (kb) is eluted and further amplified with 6 cycles of 95°C for 1 minute, 58°C for 1 minute and 72°C for 5 minutes. This material is further separated on a 2% Nu-sieve agarose gel, and the material running from 6kb on the gel is eluted and further amplified using the same PCR conditions for 27 cycles. The amplified DNA derived from this high molecular weight region is then further fractionated on a 2 % Nu-Sieve gel, and cDNA from 0.5 to 1.5kb, and from 1.5 to 5kb pooled.

1.3. **Library Construction**

10µg of the bacteriophage vector lambda-zap II (Stratagene) is restriction digested with NotI and EcoRI in high salt buffer overnight at 37°C followed by dephosphorylation using 1 unit of calf intestinal phosphatase (Promega) for 30 minutes at 37°C in 10mM Tris.HCl (pH9.5), 1mM spermidine, 0.1mM EDTA. DRG cDNA is digested with Klenow enzyme in the presence of dGTP and dCTP to construct an EcoRI site from the oligo-dC primer (see above) at the 5' end of the cDNA, and cut with NotI for directional cloning. The cDNA is ligated into the cloning vector bacteriophage lambda-zap II for 16 hours at 12°C. Recombinant phage DNA is then packaged into infective phage using Gigapack gold (Stratagene) and protocols specified by the suppliers. 0.1% of the packaged DNA is used to infect E.coli BB4 cells which are plated out to calculate the number of independent clones generated.

1.4 Differential Screening

The library is plated at a low density (10^3 clones/ 12 x 12 cm^2 dish) and screened using three sets of ^{32}P-labelled cDNA probes and multiple filter lifts. Replica filters are made by laying them onto the plated library plates, briefly drying them and then laying onto fresh agar plates to increase the quantity of phage and the subsequent hybridisation signals of lifts taken from them. The probes are derived from: a) cortex and cerebellum poly (A)+ RNA, b) DRG poly (A)+ RNA , and c) subtracted cDNA from DRG. The two mRNA probes are labelled with ^{32}P dCTP using a reaction mixture containing 2-5µg RNA, 50µl 5 x RT buffer, 25 µl 0.1M dithiothreitol (DTT), 12.5µl

10mM dATP, dGTP, dCTP, 30pM oligo-dT, 75 µl ^{32}P-dCTP (30MBq; Amersham), 25µl 100µM dCTP, 2µl RNasin (2units/µl) and 2µl SuperScript reverse transcriptase (GibcoBRL) in a final volume of 250µl. The reaction is incubated at 39°C for 60 minutes, and the RNA subsequently destroyed by adding 250µl water, 55µl 1M NaOH, and incubating at 70°C for 20 minutes. The reaction mixture is neutralised with acidified Tris base (pH 2.0) and precipitated with carrier tRNA (Boehringer) with isopropanol. The subtracted and amplified double-stranded DRG cDNA is random-prime labelled with ^{32}P dATP (Gibco multiprime kit). Replica filters are then prehybridised for 4 hours at 68°C in hybridisation buffer. Hybridisation was carried out for 20 hours at 68°C in 4x SSC (20xSSC consists of 175.3g of sodium chloride and 88.2g of sodium citrate in 800ml of distilled water. The pH is adjusted to 7.0 with 10N sodium hydroxide and this is made to 1 litre with distilled water), 5x Denhardts solution containing 150 µg/ml salmon sperm DNA, 20µg/ml poly-U, 20µg/ml poly-C, 0.5% SDS (Sigma), 5mM EDTA. The filters are briefly washed in 2 x SSC at room temperature, then twice with 2 x SSC with 0.5% SDS at 68°C for 15 minutes, followed by a 20 minute wash in 0.5% SDS, 0.2 x SSC at 68°C. The filters are autoradiographed for up to 1 week on Kodak X-omat film. Plaques that hybridise with DRG probes but not cortex and cerebellum probes are picked, phage DNA prepared and the cloned inserts released for subcloning into pBluescript (Stratagene).

The positive plaques are picked by lining up the autoradiogram with the plate using orientation marks and taking a plug from the plate corresponding to the positive hybridisation signal. The phage is eluted from the plug in 0.5ml phage dilution buffer (10mM Tris chloride (pH7.5) 10mM magnesium sulphate) and the phage re-infected into E.coli BB4 and replated at a density of 200 to 1000 plaques/150mm plate as a secondary purification step to ensure purity of the clones. The positive secondaries are then picked as described previously. In order to sub-clone the insert DNA from the positive recombinant phage, they need to be amplified. This is accomplished by plate lysis where the phage totally lyse the E.coli BB4. 0.2ml of phage suspension is mixed with 0.1ml of an overnight culture of E.coli. This is added to 2.5ml of top agar (16g bacto-tryptone 10g bacto-yeast extract, 5g sodium chloride, 7g bacto-agar in 900mls distilled water) and plated onto 9cm^2 agar plates. These are incubated overnight at 37°C. 5ml of phage dilution buffer is then added to the plates and is incubated overnight at 4°C or for 4 hours with gentle scraping at

room temperature. The phage-containing buffer is then recovered, 0.1ml chloroform is added and this phage stock is titrated as above and stored at 4°C. Phage DNA is prepared by first infecting 10^{10} E.coli B44 with 10^9 plaque forming units (pfus) of phage in 3ml of phage dilution buffer and shaking at 37°C for 20 minutes. The infected bacteria are added to 400ml of L broth (1.6% bactotryptone, 0.5% (w/v) Bacto yeast extract, 0.5% (w/v) magnesium sulphate) with vigorous shaking at 37°C for 9 hours. When lysis has occurred, 10ml of chloroform is added and shaking is continued for a further 30 minutes. The culture is then cooled to room temperature and pancreatic RNAase and DNAase are added to 1ug/ml for 40 minutes. Sodium chloride is then added to 1M and is dissolved by swirling on ice. After centrifuging at 8000rpm for 10 minutes the supernatant is recovered. Polyethylene glycol (PEG 6000) is added to 10% w/v and is dissolved by stirring whilst on ice for 2 hours. After centrifuging for 8000rpm for 10 minutes at 4°C the pellet is resuspended in 8ml of phage dilution buffer. This is extracted with an equal volume of phenol/chloroform followed by purification on a caesium chloride gradient (0.675g/ml caesium chloride - 24 hours at 38000 rpm at 4°C). The opaque phage band is removed from the centrifugation tube and dialysed against 10mM sodium chloride, 50mM Tris (pH8.0), 10mM magnesium chloride for 2 hours. EDTA is then added to 20mM, proteinase K to 50µg/ml and SDS to 0.5% and is incubated at 65°C for 1 hour. After dialysis overnight against TE pure phage DNA results. The cloned insert is digested from the purified phage DNA using restriction enzymes as previously described. Each phage insert is then ligated into a plasmid vector e.g. pBluescript - Clontech using a ligation reaction as previously described.

Clone characterisation.

The plasmids are cross hybridised with each other. Unique clones are further analysed by Northern blotting and sequencing. The clone/s showing transcript sizes and sequence comparable with sodium channels are then used as hybridisation probes to screen a neonatal rat DRG oligo dT-primed full length cDNA library to derive full length cDNA clones using methodology as described above and in example 2. Biological activity of the rat DRG sodium channel is confirmed as in examples 4 and 7 below.

Example 2 - Homology cloning of the human cDNA homologous to the rat DRG sodium channel cDNA (SNS-B).

2.1. Isolation of human ganglia total RNA

The starting material for the derivation of the human cDNA homologue of the rat DRG sodium channel cDNA is isolated human dorsal root ganglia or trigeminal ganglia or other cranial ganglia from post-mortem human material or foetuses. Total ribonucleic acid (RNA) is isolated from the human neural tissue by extraction in guanidinium isothiocyanate (Chomczynski and Sacchi 1987 Anal Biochem 162,156-159) as described in example 1.

2.2 Determination of the transcript size of the human homologue of the rat DRG sodium channel cDNA (SNS-B).

Human dorsal root ganglia total RNA is electrophoretically separated in a 1% (w/v) agarose gel containing a suitable denaturing agent e.g. formaldehyde (Lehrach et al 1977 Biochemistry 16,4743; Goldberg 1980 Proc Natl Acad Sci 77,5794; Seed 1982 in Genetic engineering: principles and methods (ed JK Setlow and A Hollaender) vol 4 p91 Plenum Publishing New York) or glyoxal/DMSO (McMaster GK and Carmichael GG 1977 Proc Natl Acad Sci 74,4835), followed by transfer of the RNA to a suitable membrane (e.g. nitrocellulose). The immobilised RNA is then hybridised to radioactive (or other suitable detection label) probes consisting of portions of the rat sodium channel cDNA sequence (see below). After washing of the membrane to remove non-hybridised probe, the hybridised probe is visualised using a suitable detection system (e.g. autoradiography for ^{32}P labelled probes) thus revealing the size of the human homologous mRNA molecule. Specifically, 20-30 µg total RNA from neonatal rat tissues are separated on 1.2% agarose -formaldehyde gels, and capillary blotted onto Hybond-N (Amersham) (Ninkina et al. 1993 Nuc Ac Res 21,3175-3182). The amounts of RNA on the blot are roughly equivalent, as judged by ethidium bromide staining of ribosomal RNA or by hybridisation with the ubiquitously expressed L-27 ribosomal protein transcripts (Le Beau et al. 1991 Nuc Ac Res 19,1337). Each Northern blot contains human DRG, cortex, cerebellum, liver kidney, spleen and heart RNA. Probes (50ng) are labelled with ^{32}P-dATP

(Amersham) by random priming. Filters are prehybridised in 50% formaldehyde 5 x SSC containing 0.5% SDS, 5 x Denhardts solution (50x Denhardts contains 5g of Ficoll (Type 400, Pharmacia), 5g of polyvinylpyrrolidone, 5g of bovine serum albumin (Fraction V, Sigma) and water to 500ml), 100 µg/ml boiled salmon sperm DNA, 10 µg/ml poly-U and 10 µg/ml poly-C at 45°C for 6 hours. After 36 hours hybridisation in the same conditions, the filters are briefly washed in 2 x SSC at room temperature, then twice with 2 x SSC with 0.5% SDS at 68°C for 15 minutes, followed by a 20 minute wash in 0.5% SDS, 0.2 x SSC at 68°C. The filters are autoradiographed for up to 1 week on Kodak X-omat film. The transcript size is calculated from the signal from the gel in comparison with gel molecular weight standard markers.

2.3 Production of a human DRG cDNA library

In order to produce a representative cDNA library from the human dorsal root ganglia messenger RNA (poly A+ mRNA) is first isolated from the total RNA pool using oligo-dT cellulose chromatography (Aviv and Leder 1972 Proc Natl Acad Sci 69,1408-1411) using methodology described in example 1. Synthesis of the first strand of cDNA from the polyA+ RNA uses the enzyme RNA-dependent DNA polymerase (reverse transcriptase) to catalyse the reaction. The most commonly used method of second strand cDNA synthesis uses the product of first strand synthesis, a cDNA:mRNA hybrid, as a template for priming the second strand synthesis. (Gubler and Hoffman 1983 Gene 25,263)).

2.3.1. First strand cDNA synthesis

20µg of human DRG polyA+ RNA is pre-treated to destroy secondary structure which may inhibit first strand cDNA synthesis. 20µg of polyA+ RNA, 1µl 1M Tris (pH7.5) are made up to a volume of 100µl with distilled water. This is incubated at 90°C for 2 minutes followed by cooling on ice. 4.8 µl of 100 mM methyl mercury is then added for 10 minutes at room temperature. 10µl of 0.7M ß-mercaptoethanol and 100 units of human placental RNAase inhibitor are then added for 5 minutes at room temperature.

The first strand synthesis reaction consists of 8µl 20mM dATP, 5µl 20mM dCTP, 8µl 20mM dGTP 8µl 20mM dTTP, 10µl 1mg/ml oligo-dT (12-18), 20µl 1M Tris (pH 8.3) (at 45°C), 8µl 3M potassium chloride, 3.3µl 0.5M magnesium chloride, 3µl $a^{32}P$ dCTP, 100 units Superscript II reverse transcriptase (GibcoBRL) made up to 200µl with distilled water. This reaction mixture is incubated at 45°C for 45 minutes after which another 50 units of Superscript reverse transcriptase is added and incubated for a further 30 minutes at 45°C. EDTA is then added to 10mM to terminate the reaction and a phenol/chloroform extraction is carried out. The DNA is then precipitated using ammonium acetate (freezing in dry ice/ethanol before centrifuging), washed with 70% ethanol and resuspended in 50ml distilled water. The size of the single stranded DNA is assessed by electrophoretically separating it out on an agarose gel (1% w/v) and autoradiographing the result against markers.

2.3.2 Second strand synthesis

The second strand synthesis reaction mixture consists of 0.5µg human DRG single stranded DNA, 2µl 1M Tris (pH7.5), 1µl 0.5M magnesium chloride, 3.33µl 3M potassium chloride, 2µl 0.5M ammonium sulphate, 1.5µl 10mM ßnicotinamide adenine dinucleotide (NAD), 4µl of each of the 1mM dNTPs, 5µl 1mg/ml bovine serum albumin (BSA), 1 unit RNAase-H, 25 units Klenow polymerase all made up to 100µl with distilled water. This is incubated at 12°C for 1 hour and then at 20°C for 1 hour. The reaction is stopped by addition of EDTA to 20mM followed by a phenol/chloroform extraction. The DNA is ethanol precipitated (-70°C overnight) and is then washed with 70% ethanol followed by resuspension in 20µl distilled water. Size is checked by gel electrophoresis and autoradiography.

2.3.3 Double stranded cDNA end repair

In order to add linkers to the end of the cDNA molecules for subsequent cloning, the ends must first be repaired. The human DRG cDNA is treated with 500 units/ml of S1 nuclease in 0.25M sodium chloride, 1mM zinc sulphate, 50mM sodium

acetate (pH4.5). Incubation is at 30°C for 40 minutes followed by neutralisation with Tris (pH 8.0) to 0.2M. The DNA is again ethanol precipitated, washed in 70% ethanol and resuspended in 20ul distilled water. The size is again checked to ensure that S1 nuclease digestion has not radically reduced the average DNA fragment size. The repair reaction consists of 19µl cDNA, 3µl 10xT4 polymerase buffer (0.33M Tris acetate (pH7.9), 0.66M potassium acetate, 0.1M magnesium acetate, 1mg/ml BSA and 5mM DTT), 2µl of each dNTP at 2mM, 2µl T4 polymerase and 4µl distilled water. This is incubated at 37°C for 30 minutes followed by addition of 1µl Klenow polymerase for 1 hour at room temperature. The DNA is then ethanol precipitated, washed in 70% ethanol and resuspended in 5µl distilled water. In order to protect naturally occurring restriction sites within the cDNA from being cleaved, the cDNA is treated with a methylase before the addition of linkers. The reaction mixture consists of 5µl human DRG double stranded DNA, 1µl S-adenosylmethionine, 2µl 1mg/ml BSA, 2µl 5x methylase buffer (0.5M Tris (pH8.0), 5mM EDTA), 0.2µl EcoRI methylase (NEB). This is incubated at 37°C for 20 minutes followed by phenol extraction, ethanol precipitation washing with 70% ethanol and resuspension in 20µl distilled water.

2.3.4. Addition of linkers to cDNA

EcoRI linkers are ligated to the cDNA molecules to facilitate cloning into lambda vectors. The ligation reaction mixture consists of 1µl 10x ligation buffer (0.5M Tris chloride (pH7.5), 0.1M magnesium chloride and 0.05M DTT), 1µl 10mM ATP, 100ng cDNA, 5µg EcoRI linkers, 1 unit T4 DNA ligase, distilled water to 10µl. The reaction is incubated at 37°C for 1 hour, followed by addition of 6 more units of T4 ligase and a further incubation overnight at 15°C. The ligated samples are ethanol precipitated, washed in 70% ethanol and resuspended in 10µl distilled water. The cDNA is then digested with EcoRI to cleave any linker concatamers formed in the ligation process. This restriction digestion reaction contains 10µl cDNA, 2µl high salt buffer (10mM magnesium chloride, 50mM Tris chloride (pH7.5), 1mM DTT, 100mM sodium chloride), 2µl EcoRI (10 units/µl - NEB) and distilled water to 20µl. The digestion is carried out for 3 hours. The ligation

and digestion steps are monitored using gel elecrophoresis to monitor the size of the products.

2.3.5 Size fractionation of cDNA

In order to assure that the library is not swamped with short cDNA molecules and to remove linker molecules a column purification is carried out. A 1ml Sepharose 4B column is made in a 1 ml plastic pipette plugged with a small piece of glass wool. This is equilibrated with 0.1M sodium chloride in TE. The cDNA is loaded onto the column and 1 drop fractions are collected. 2µl aliquots of each fraction are analysed by gel electrophoresis and autoradiography to determine the sizes of the cDNA in each fraction. Fractions containing cDNA of about 800 base pairs and above are pooled and purified by ethanol precipitation and resuspending in 10µl distilled water.

2.3.6 Cloning of cDNA into bacteriophage vector

Bacteriophage vectors designed for the cloning and propagation of cDNA are provided ready-digested with EcoRI and with phosphatased ends from commercial sources (e.g. lambda gt10 from Stratagene). The prepared subtracted cDNA is ligated into lambda gt10 using a ligation rection consisting of ligase buffer and T4 DNA ligase (New England Biolabs) as described elsewhere in this document.

2.4 Labelling of cDNA fragments (probes) for library screening

The 3' untranslated region of the rat DRG sodium channel cDNA clone (SNS-B) is subcloned using appropriate restriction enzymes into a plasmid vector e.g. pBluescript - Stratagene. The cDNA insert which is to form the labelled probe is released from the vector via digestion with appropriate restriction enzymes and the insert is separated from the vector via electrophoresis in a 1% (w/v) agarose gel. After removal of the separated insert from the agarose gel and purification it is labelled by standard

techniques such as random priming and polymerisation (Feinberg and Vogelstein 1983 Anal Biochem 132,6) or nick translation (Rigby et al 1977 J Mol Biol 113,237) with ^{32}P or DIG-labelled nucleotides. Alternatively, if the probe cDNA insert is cloned into a vector containing strong bacteriophage promoters to which DNA-dependant RNA polymerases bind (SP6, T3 or T7 polymerases), synthetic cRNA is produced by in vitro transcription which incorporates ^{32}P or digoxygenin nucleotides. Other regions of the rat DRG sodium channel cDNA can also be used as probes in a similar fashion for cDNA library screening or Northern blot analysis. Specifically, a probe is made using a kit such as the Pharmacia oligo labelling kit. This will radioactively label the rat DRG sodium channel cDNA fragment. 50ng of denatured DNA (place in boiling waterbath for 5 minutes), 3µl of ^{32}PdCTP (Amersham) and 10µl reagent mix is made up to 49µl with distilled water. 1µl of Klenow fragment is added and the mixture is incubated at 37°C for one hour. To remove unincorporated nucleotides, the reaction mixture is applied to a Nick column (Sephadex G50 - Pharmacia) followed by 400µl of TE (10mM Tris chloride (pH7.4) 1mM EDTA (pH8.0)). Another 400µl of TE is added and the eluate is collected. This contains the labelled DNA to be used as a hybridisation probe.

2.5 cDNA library screening

In order to detect recombinants containing human homologues of the rat DRG sodium channel the human DRG cDNA library is screened using moderate stringency hybridisation washes (50-60°C, 5 x SSC, 30 minutes), using radiolabelled or other labelled DNA or cRNA probes derived from the 3' untranslated region as described above. Libraries are screened using standard methodologies involving the production of nitrocellulose or nylon membrane replicas of DNA from recombinant plaques formed on agar plates (Benton et al 1977 Science 196;180). These are then hybridised to single stranded nucleic acid probes (see above). Moderate stringency washes are carried out (see wash conditions for Northern analysis in section 2.2). Plaques which are positive on duplicate filters (i.e. not artefacts or background) are then purified by one or more rounds of replating after dilution to separate the colonies and further hybridisation screening. Resulting positive plaques are purified, DNA is extracted and the insert sizes of these

clones is examined. The clones are cross-hybridised to each other using standard techniques (Sambrook et al 1989 Molecular Cloning Second Edition Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) and distinct positive clones identified. Detailed protocols for cDNA library screening are given in example 1.

2.6 Derivation of a full-length clone of the human homologue of the rat DRG sodium channel cDNA

Overlapping positive clones from above are identified by cross-hybridisation. They are then restriction mapped to identify their common portions and restriction fragments representing the separate portions from the overlapping clones are ligated together using standard cloning techniques (Sambrook et al 1989 Molecular Cloning Second Edition Cold Spring Harbor Laboratory Press). For example, the most 5' fragment will contain any 5' untranslated sequence, the start codon ATG and 5' coding sequence. The most 3' clone will contain the most 3' coding sequence, a stop codon and any 3' untranslated sequence, a poly A consensus sequence and possibly a poly A run. Thus a recombinant molecule is generated which contains the full cDNA sequence of the human homologue of the rat DRG sodium channel cDNA. If overlapping clones do not produce sufficient fragments to assemble a full length cDNA clone, the full length oligo dT-primed human DRG library is re-screened to isolate a full length clone. Alternatively, a full length clone is derived directly from the library screening.

2.7 Characterisation of the human homologue full-length clone

The cDNA sequence from the full-length clone is used as a probe in Northern blot analysis to detect the messenger RNA size in human tissue for comparison with the rat messenger RNA size (see sections 1.1 and 2.2 for methodology).

Confirmation of biological activity of the cloned cDNA is carried out via in vitro translation of the human sodium channel mRNA and its expression in Xenopus oocytes in an analogous manner to that for the rat DRG-specific TTXi resistant sodium channel as described in examples 4 and 7.

cDNA sequences which are shown to have activity as defined above are completely sequenced using dideoxy-mediated chain termination sequencing protocols (Sanger et al 1977 Proc Natl Acad Sci 74,5463).

Example 3 - Polymerase chain reaction (PCR) approaches to clone the human DRG sodium channels using DNA sequence derived from the rat DRG sodium channel cDNA clone

Total RNA and poly A+ RNA is isolated from human dorsal root ganglia or trigeminal ganglia or other cranial ganglia from post-mortem human material or foetuses as described in example 2 above.

Random primers are hybridised to the RNA followed by polymerisation with MMLV reverse transcriptase to generate single stranded cDNA from the extracted human RNA.

Using degenerate PCR primers derived from relatively conserved regions of the known voltage-gated sodium channels (Figure 2), amplify the cDNA using the polymerase chain reaction (Saiki et al 1985 Science 230,1350). It is appreciated by those skilled in the art that there are many variables which can be manipulated in a PCR reaction to derive the homologous sequences required. These include but are not limited to varying cycle and step temperatures, cycle and step times, number of cycles, thermostable polymerase, Mg2+ concentration. It is also appreciated that greater specificity can be gained by a second round of amplification utilising one or more nested primers derived from further conserved sequence from the sodium channels.

Specifically, the above can be accomplished in the following manner. The first strand cDNA reaction consists of 1µg of total RNA made up to 13µl with DEPC-treated water and 1µl of 0.5µg/µl oligo(dT). This is heated to 70°C for 10 minutes and then incubated on ice for 1minute. The following is then added: 2µl of 10x synthesis buffer (200mM Tris chloride, 500mM potassium chloride, 25mM magnesium chloride, 1µg/ml BSA), 2µl of 0.1M DTT, 1µl of 200U/µl Superscript Reverse Transcriptase (Gibco BRL). This is incubated at room temperature for 10 minutes then at 42°C for 50 minutes. The reaction is then terminated by incubating for 15 minutes at 70°C. 1µl of E.coli RNase H (2U/µl) is added to the tube which is then incubated for 20 minutes at 37°C.

The PCR reaction is set up in a 0.5ml thin-walled Eppendorf tube. The following reagents are added: 10µl 10x PCR buffer, 1µl cDNA,16µl dNTP's (25µl of 100µM dATP,dCTP, dCTP and dGTP into 900µl sterile distilled water), 7µl of 25mM magnesium chloride, 1µl of Taq DNA polymerase (Amplitaq Perkin-Elmer)plus sterile distilled water to 94µl.

To each reaction tube a wax PCR bead is added (Perkin-Elmer) and the tube placed in a 70°C hot block for 1 minute. The tubes are allowed to cool until the wax sets and 3µl of each primer (33pM/µl) are added above the wax. The tubes are placed in a thermal cycler (Perkin-Elmer) and the following 3-step program used after an initial 94°C for 5 minutes; 92°C for 2 minutes, 55°C for 2 minutes, 72°C for 2 minutes for 35 cycles. A final polymerisation step is added at 72°C for 10 minutes. The reaction products are then run on a 1% agarose gel to assess the size of the products. In addition, control reactions are performed alongside the samples. These should be: 1) all components without cDNA (negative control) and 2) all reaction components with primers for constitutively expressed product e.g. α-actin or HPRT.

The products of the PCR reactions are examined on 0.8%-1.2% (w/v) agarose gels. Bands on the gel (visualised by staining with ethidium bromide and viewing under UV light) representing amplification products of the approximate predicted size were then cut from the gel and the DNA purified. Further bands of interest are also identified by Southern blot analysis of the amplification products and probing of the resulting filters with labelled primers from further conserved regions e.g. those used for secondary amplification.

The resulting DNA is ligated into suitable vectors such as, but not limited to, pCR II (Invitrogen) or pGemT. Clones are then sequenced to identify those containing sequence with similarity to the rat DRG sodium channel sequence (SNS-B).

Clone analysis

Candidate clones from above are used to screen a human cDNA DRG library constructed using methods described in example 2. If a full length clone is not identified, positive overlapping clones which code for the full length human cDNA

homologue are identified and a full length clone is then assembled as described in example 1. Biological activity is then confirmed as described in examples 4 and 7.

Example 4 - In vitro translation of rat and human DRG sodium channel in Xenopus laevis oocytes

In order to demonstrate the biological activity of the protein coded for by the rat DRG sodium channel cDNA sequence (SNS-B) and its human homologue the complete double-stranded cDNA coding sequences are ligated into in vitro transcription vectors (including but not limited to the pGEM series, Promega) using one or more of the available restriction enzyme sites such that the cDNAs are inserted in the correct orientation. The constructs are then used to transform bacteria and constructs with the correct sequence in the correct orientation are identified via diagnostic restriction enzyme analysis and dideoxy-mediated chain termination DNA sequencing (Sanger et al 1977 Proc Natl Acad Sci 74,5463).

These constructs are then linearised at a restriction site downstream of the coding sequence and the linearised and purified plasmids are then utilised as a template for in vitro transcription. Sufficient quantities of synthetic mRNA are produced via in vitro transcription of the cloned DNA using a DNA-dependent RNA polymerase from a bacteriophage that recognises a bacteriophage promoter found in the cloning vector. Examples of such polymerases include (but are not limited to) T3, T7 and SP6 RNA polymerase.

A variation on the above method is the synthesis of mRNA containing a 5' terminal cap structure (7-methylguanosine) to increase its stability and enhance its translation efficiency (Nielson and Shapiro 1986 Nuc Ac Res 14,5936). This is accomplished by the addition of 7-methylguanosine to the reaction mixture used for synthetic mRNA synthesis. The cap structure is incorporated into the 5' end of the transcripts as polymerisation occurs. Kits are available to facilitate this process e.g. mCAP RNA Capping Kit - Stratagene).

The synthetic RNA produced from the in vitro transcription is isolated and purified. It is then translated via microinjection into Xenopus laevis oocytes. 50nls of 1mg/ml synthetic RNA is micro-injected into stage 5 or stage 6 oocytes according to methods established in the literature (Gurdon et al (1983) Methods in Enzymol 101,370).

After incubation to allow translation of the mRNAs the oocytes are analysed for expression of the DRG sodium channels via electrophysiological or other methods as described in example 7.

A further method for expression of functional sodium channels involves the nuclear injection of a Xenopus oocyte protein expression vector such as pOEV (Pfaff et al., Anal. Biochem. 188, 192-195 (1990)) which allows cloned DNA to be transcribed and translated directly in the oocyte. Since proteins translated in oocytes are post-translationally modified according to conserved eukaryotic signals, these cells offer a convenient system for performing structural and functional analyses of cloned genes. pOEV can be used for direct analysis of proteins encoded by cloned cDNAs without preparing mRNA in vitro, simplifying existing protocols for translating proteins in oocytes with a very high translational yield. Transcription of the vector in oocytes is driven by the promoter for the TFIIIA gene, which can generate 1-2 ng (per oocyte within 2 days) of stable mRNA template for translation. The vector also contains SP6 and T7 promoters for in vitro transcription to make mRNA and hybridization probes. DNA clones encoding SNS channel transcripts are injected into oocyte nuclei and protein accumulated in the cell over a 2- to 10-day period. The presence of functional protein is then assessed using twin electrode voltage clamp as described in example 7.

Example 5 - Expression of rat and human DRG sodium channel in mammalian cells

In order to be able to establish a mammalian cell expression system capable of producing the sodium channel in a stable bioactive manner, constructs have to be first generated consisting of the cDNA of the channel in the correct vectors suitable for the cell system in which it is desired to express the protein. There are available a range of vectors containing strong promoters which drive expression in mammalian cells.

i/ Transient expression

In order to determine rapidly the bioactivity of a given cDNA it can be introduced directly into cells and resulting protein activity assayed 48-72 hours later. Although this does not result in a cell line which is stably expressing the protein of interest

it does give a quick answer as to the biological activity of the molecule. Specifically, the cDNA representing the human or rat DRG sodium channel is ligated into appropriate vectors (including but not limited to pRc/RSV, pRc/CMV, pcDNA1 (Invitrogen)) using appropriate restriction enzymes such that the resulting construct contains the cDNA in the correct orientation and such that the heterologous promoter can drive expression of the transcription unit. The resulting expression constructs are introduced into appropriate cell lines including but not limited to COS-7 cells (an African Green Monkey Kidney cell line), HEK 293 cells (a human embryonic kidney cell line) and NIH3T3 cells (a murine fibroblastic cell line). The DNA is introduced via standard methods (Sambrook et al 1989 Molecular Cloning Second Edition, Cold Spring Harbour Laboratory Press) including but not limited to calcium phosphate transfection, electroporation or lipofectamine (Gibco) transfection. After the required incubation time at 37°C in a humidified incubator the cells are tested for the presence of an active rat DRG sodium channel using methods described in example 7.

ii/Stable expression

The production of a stable expression system has several advantages over transient expression. A clonal cell line can be generated that a has a stable phenotype and in which the expression levels of the foreign protein can be characterised and , with some expression systems, controlled. Also, a range of vectors are available which incorporate genes coding for antibiotic resistance, thus allowing the selection of cells transfected with the constructs introduced. Cell lines of this type can be grown in tissue culture and can be frozen down for long-term storage. There are several systems available for accomplishing this e.g. CHO, CV-1, NIH-3T3.

Specifically COS-7 cells can be transfected by lipofection using Lipofectamine (GibcoBRL) in the following manner. For each sample $2x10^6$ cells are seeded in a 90mm tissue culture plate the day prior to transfection. These are incubated overnight at 37°C in a CO_2 incubator to give 50-80% confluency the following day. The day of the transfection the following solutions are prepared in sterile 12 x 75mm tubes: Solution A: For each transfection, dilute 10-50µg of DNA into 990µl of serum-free media (Opti-MEM I Reduced Serum Medium GibcoBRL). Solution B: For each transfection,

dilute 50µl of Lipofectamine Reagent into 950µl serum-free medium. The two solutions are combined, mixed gently and incubated at room temp for 45 minutes. During this time the cells are rinsed once with serum-free medium. For each transfection 9ml of serum-free medium is added to the DNA-lipofectamine tubes. This solution is mixed gently and overlayed on the rinsed cells. The plates are incubated for 5 hours at 37°C in a CO_2 incubator. After the incubation the medium is replaced with fresh complete media and the cells returned to the incubator. Cells are assayed for activity 72 hours post transfection as detailed in examples 4 and 7. To ascertain the efficiency of transfection, ß-galactosidase in pcDNA3 is transfected alongside the DRG sodium channel cDNA. This control plate is stained for ß-galactosidase activity using a chromogenic substrate and the proportion of cells staining calculated. For transient transfection of DRG the cDNA must first be cloned into a eucaryotic expression vector such as pcDNA3 (Invitrogen).

Example 6 - Expression of rat DRG sodium channel in insect cells

The baculovirus expression system uses baculovirus such as Autographa californica nuclear polyhedrosis virus (AcNPV) to produce large amounts of target protein in insect cells such as the Sf9 or 21 clonal cell lines derived from Spodoptera frugiperda cells. Expression of the highly abundant polyhedrin gene is non-essential in tissue culture and its strong promoter (polh) can be used for the synthesis of foreign gene products (Smith et al 1983 Mol Cell Biol 3,2156-2165). The polyhedrin promoter is maximally expressed very late in infection (20 hours post infection).

A transfer vector, where the rat DRG sodium channel cDNA is cloned downstream of the polh promoter, or another late promoter such as p10, is transfected into insect cells in conjunction with modified AcNPV viral DNA such as but not limited to BaculoGold DNA (PharMingen). The modified DNA contains a lethal mutation and is incapable of producing infectious viral particles after transfection. Co-transfection with a complementing transfer vector such as (but not limited to) pAcYM1 (Matsuura et al 1987 J Gen Virol 68,1233-1250) or pVL1392/3 (InVitrogen) allows the production of viable recombinant virus. Although more than 99% of the resultant virus particles should be derived from plasmid-rescued virus it is desirable to further purify the virus particles by plaque assay. To ensure that the recombinant stock is clonal, a single plaque is picked from

the plaque assay and amplified to produce a recombinant viral stock. Once the recombinant phenotype is verified the viral stock can be used to infect insect cells and express functional rat DRG sodium channel. There are a number of variations in the methodology of baculovirus expression which may give increased expression (O'Reilly et al 1992 Baculovirus Expression Vectors: A Laboratory Manual. Oxford University Press). The expression of the rat or human DRG sodium channel is achieved by cloning of the cDNA into pVL1392 and introducing this into Sf21 insect cells.

Example 7 - Electrophysiological characterisation of cloned human and rat DRG sodium channel expression

Xenopus laevis oocytes are used to express the channel after injection of the mRNA or cDNA in an expression vector. Expression would be transient and thus functional studies would be made at appropriate times after the injections. Comparison with mock-injected oocytes would demonstrate lack of the novel channel as an endogenously expressed characteristic. Standard two electrode voltage clamp (TEVC) techniques as described, for example, in Fraser, Moon & Djamgoz (1993) Electrophysiology of *Xenopus* oocytes: an expression system in molecular neurobiology. In: Electrophysiology: A practical approach. Wallis, D.I., ed. Oxford University Press. Chapter 4 pp. 65-86, would be used to examine the characteristics of responses of ionic currents to changes in the applied membrane potential. Appropriately modified saline media would be used to manipulate the type of ionic currents detectable. The kinetics of activation and inactivation of the sodium current, its ionic selectivity, the effects of changes in ionic concentration of the extracellular medium on its reversal potential, and the sensitivity (or resistance) to TTX would be defining characteristics.

Similar electrophysiological studies would be undertaken to assess the success of functional expression in a permanently or transiently expressing mammalian cell line, but patch clamp methods would be more suitable than TEVC. Whole cell, cell-attached patch, inside-out patch or outside-out patch configurations as described for example by Hamill et al. (1981) Pflugers Arch. 391:85-100 and Fenwick et al. (1982) J. Physiol. 331 599-635 might be used to assess the channel characteristics.

For example, isolated transfected cells (see above) will be voltage-clamped using the whole-cell variant of the patch clamp technique for recording the expressed sodium channel current.

Recordings will be obtained at room temperature (22-24°C). Both external and internal recording solutions will be used to isolate Na+ currents as previously described (Lalik et al., Am. J. Physiol. 264:C803-C809, 1992; West et al., Neuron 8:59-70, 1992). External solution (mM): sodium chloride, 65; choline chloride, 50; TEA-Cl, 20, KCl, 1.5; calcium chloride, 1; magnesium chloride, 5; glucose 5; HEPES, 5; at a pH 7.4 and and osmolality of 320. Internal solution (mM):CsF, 90; CsCl, 60; sodium chloride, 10; MgCl, 2;EGTA, 10; HEPES, 10 at pH 7.2 and an osmolarity of 315.

The kinetics and voltage parameters of the expressed sodium channel current will be examined and compared with data existing in the literature. These include current-voltage relationships and peak current amplitude. Cells will be voltage-clamped at -70 mV and depolarizing pulses to 50 mV (at 10 mV increments) will be used to generate currents.

The pharmacology of the expressed sodium channel current will be examined with the Na channel blocker, tetrodotoxin (TTX). To date sodium channels have been classified as TTX-sensitive and TTX-resistant: block by low (1-30 nM) and high (> 1 μM) concentrations of TTX, respectively (Elliot & Elliot, J. Physiol. (Lond.) 463:39-56, 1993; Yang et al., J. Neurosci. 12:268-277, 1992; W1992).

The channel is unaffected by concentrations lower than 1 micromolar tetrodotoxin, and is only partially blocked by concentrations as high as 10 micromolar tetrodotoxin.

Example 8 - Production of purified channel

Using a commercial coupled transcription-translation system, 35-S methionine labelled protein products of the SNS clone can be generated (see **Figure 3**). The size of the resulting protein when assessed by SDS-polyacrylamide gel electrophoresis confirms the predicted size of the protein deduced by DNA sequencing. The system used

is the Promega TNT system (Promega Technical Bulletin 126 1993). The experiment is carried out precisely according to the protocol provided (see **Figure 3**).

Example 9 - Use of rat or human sodium channel in screening assays

Cell lines expressing the cloned sodium channels could be used to determine the effects of drugs on the ability of the channels to pass sodium ions across the cell membranes, e.g to block the channels or to enhance their opening. Since the channel activation is voltage dependent, depolarising conditions will be required for observation of baseline activity that would be modified by drug actions. Depolarisation could be achieved by for example raising extracellular potassium ion concentration to 20 or 40 mM, or by repeated electrical pulses. Detection of the activation of sodium conducting channels could be achieved by flux of radiolabelled sodium ions, guanidine or by reporter gene activation leading to for example a colour change or to fluorescence of a light emitting protein. Subsequent confirmation of the effectiveness of the drug action on sodium channel activity would require electrophysiological studies similar to those described above.

Example 10 - In vitro influx assays

1. 22Na+ influx assay: A modified assay has been adapted from methods reported by Tamkum and Catterall, Mol Pharm. 19:78, (1981). Oocytes or cells expressing the sodium channel gene are suspended in a buffer containing 0.13 M sodium chloride, 5 mM KCl, 0.8 mM $MgSO_4$, 50 mM HEPES-Tris (pH 7.4), and 5.5 mM glucose. Aliquots of the cell suspension are added a buffer containing 22NaCl (1.3 μCi/ml, New England Nuclear, Boston, MA), 0.128 M choline chloride, 2.66 mM sodium chloride, 5.4 mM KCl, 0.8 mM $MgSO_4$, 50 mM HEPES-Tris (pH 7.4), 5 mM ouabain, 1mg/ml bovine serum albumin, and 5.5 mM glucose and then incubated at 37 oC for 20 sec in either the presence or absence of 100 μM veratridine (Sigma Chemical Co., St Louis, MO). The influx assay is stopped by the addition of 3 ml of ice-cold wash buffer containing 0.163 M sodium chloride, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 50 mM HEPES-Tris (pH 7.4) and 1mg/ml bovine serum albumin,

collected on a glass fiber filter (Whatman GF/C), and washed twices with 3 ml of wash buffer. Radioactive incorporation is determined by with a gammacounter. The specific tetrodotoxin-resistant influx is measured by the difference in 22Na+ uptake in the absence or the presence of 10 μM transmethrin or 1 μM (+) trans allethrin. The tetrodotoxin-sensitive influx is measured by the difference in 22Na+ uptake in the absence or the presence of 1 μM tetrodotoxin (Sigma Chemical Co., St Louis, MO).

Guanidine influx: Another assay is modified from the method described by Reith, Eur. J. Pharmacol. 188:33 (1990). In this assay sodium ions are substituted with guanidinium ions. Oocytes or cells are washed twice with a buffer containing 4.74 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM KH2PO4, 1.18 mM $MgSO_4$, 22 mM HEPES (pH 7.2), 22 mM choline chloride and 11 mM glucose. The oocytes or cells are suspended in the same buffer containing 250 μM guanidine for 5 min at 19-25 oC. An aliquot of 14C-labelled guanidine hydrochloride (30-50 mCi/mmol supplied by New England Nuclear, Boston, MA) is added in the absence or presence of 10 μM veratridine, and the mixture is incubated for 3 min. The uptake reaction is stopped by filtration through Whatman GF/F filters and followed by 2 5 ml washes with ice-cold 0.9% saline. Radioactive incorporation is determined by scintillation counting.

Example 11

In order to measure the expression of sodium channels in in vitro systems, as well as to analyse distribution and relative level of expression in vivo, and to attempt to block function, polyclonal and monoclonal antibodies will be generated to peptide and protein fragments derived from SNS protein sequence shown in Figure 1.

a) Immunogens

Glutathione-sulphotransferase (GST) - fusion proteins will be constructed (Smith and Johnson Gene 67:31-40 (1988)) using PGEX vectors obtained from Pharmacia. Fusion proteins including both intracellular and extracellular loops with little homology with known sodium channels other than SNS-B will be produced. One such method involves subcloning of fragments into pGex-5X3 or pGEX 4t-2 to produce in-frame fusion

proteins encoding extracellular, intracellular or C-terminal domains as shown in detailed maps in Figure 4. The pGEX fusion vectors are transformed into E. coli XL-1 blue cells or other appropriate cells grown in the presence of ampicillin. After the cultures have reached an optical density of OD600 > 0.5, fusion protein synthesis is induced by the addition of 100 micromolar IPTG, and the cultures further incubated for 1- 4 hours. The cells are harvested by centrifugation and washed in ice cold phosphate buffered saline. The resuting pellet (dissolved in 300 microlitres PBS from each 50 ml culture) is then sonicated on ice using a 2mm diameter probe, and the lysed cells microfuged to remove debris. 50 microlitres of glutathione-agarose beads are then added to each pellet, and after gentle mixing for 2 minutes at room temperature, the beads are washed by successive spins in PBS. The washed beads are then boiled in Laemmli gel sample buffer, and applied to 10% polyacrylamide SDS gels. Material migrating at the predicted molecular weight is identified on the gel by brief staining with coommassie blue, and comparison with molecular weight markers. This material is then electroeluted from the gel and used as an immunogen as described below.

b) Antibody production

Female Balb/c mice are immunised intraperiteonally with 1-100 micrograms of GST fusion protein emulisfied in Freunds complete adjuvant. After 4 weeks, the animals will be further immunised with fusion proteins (1-100 micrograms) emulsified in Freunds incomplete adjuvant. Four weeks later, the animals will be immunised intraperitoneally with a further 1-100 micrograms of GST fusion protein emulsified with Freunds incomplete adjuvant. Seven days later, the animals will be tail bled, and their serum assessed for the production of antibodies to the immunogen by the following screen; (protocols for the production of rabbit polyclonal serum are the same, except that all injections are subcutaneous, and 10 times as much immunogen is used. Polyclonal rabbit serum are isolated from ear-vein bleeds.)

Serial ten-fold dilutions of the sera (1;100 to 1; 1000,000) in phosphate buffered saline (PBS) containing 0.5% NP-40 and 1% normal goat serum will be applied to 4% paraformaldehyde-fixed 10 micron sections of neonatal rat spinal cord previously treated with 10% goat serum in PBS, After overnight incubation, the sections are washed in

PBS, and further incubated in the dark with 1;200 FITC-conjugated F(ab)2 fragment of goat anti-mouse antibodies for 2 hours in PBS containing 1% normal goat serum. The sections are further washed in PBS, mounted in Citifluor, and examined by fluorescence microscopy. Those sera that show specific staining of laminar II in the spinal cord will be retained, and the mice generating such antibodies subsequently used for the production of monoclonal antibodies. Three weeks later, mice producing useful antibodies are immunised with GST-fusion proteins without adjuvant. After 3 days, the animals are killed, their spleens removed, and the lymphocytes fused with the thymidine kinase-negative myeloma line NS0 or equivalent, using polyethylene glycol. The fused cells from each experiment are grown up in 3 x 24 well plates in the presence of DMEM medium containing 10% fotal calf serum and hypoxanthine, aminopterin and thymidine (HAT) medium to kill the myeloma cells (Kohler and Milstein, Eur. J. Immunol 6, 511-519 (1976)). The tissue culture supernatants from wells containing hybridomas are further screened by immunofluorescence as described above, and cells from positive wells cloned by limiting dilution. Antibody from the positive testing cloned hybridomas is then used to Western blot extracts of rat dorsal root ganglia, to detemine if the antibody recognises a band of size approximately 200,000, confirming the specificity of the monoclonal antibody for the SNS sodium channel. Those antibodies directed against extracellular domains that test positive by both of these criteria will then be assessed for function blocking activity in electrophysiological tests of sodium channel function (see example 7), and in screens relying on ion flux or dye-based assays in cells lines expressing sodium channel (see examples 9 and 10).

Example 12 - Cell-type distribution of expression

In situ hybridization demonstrates the presence of SNS in a subset of sensory neurons. An SNS fragment between positions 1740 and 1960 was sub-cloned into pGem4z, and DIG-UTP labeled sense or antisense cRNA generated. Sample preparation, hybridization, and visualization of in situ hybridization with alkaline phosphatase conjugated anti-DIG antibodies was carried out exactly as described in Schaeren-Wimers N. and Gerfin-Moser A. Histochemistry 100, 431-440 (1993).

Example 13 - Electrophysiological Properties of the Rat DRG Sodium Channel Expressed in *Xenopus oocytes*

pBluescript SK plasmid containing DNA encoding the SNS sodium channel was digested to position -21 upstream of the initiator methionine using a commercially available kit (Erase a base system, Promega, Madison, Wisconsin, USA). The linearized and digested plasmid was cut with Kpn1 and subcloned into an oocyte expression vector pSp64GL (Sma-Kpnl) sites. pSP64GL is derived from pSP64.T pSP64.T was cut with Sma1-EcoR1, blunt-ended with Klenow enzyme, and recircularized. Part of the pGem 72 (+) polylinker (Smal-Kpn1-EcoR1-Xhol) was ligated into the blunt-ended Bg1 II site of pSP64.T. This vector with an altered polylinker for DNA inserts (Smal-Kpn1-EcoR1-Xhol) and linearization (Sal1-Xba 1-BamH1) was named pSP64GL. The resulting plasmid was linearized with Xbal, and cRNA transcribed with SP6 polymerase using 1 mM 7-methylGppG.

cRNA (70 ng) was injected into *Xenopus* oocytes 7-14 days before recording; immature, stage IV oocytes were chosen cause of their smaller diameter and therefore capacitance. Oocytes were impaled with 3M KCl electrodes ($\leq$1MΩ) and perfused at 3-4 ml per minute with modified Ringer solution containing 115 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 1.8 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 7.2, at temperature of 19.5 - 20.5 °C. Digital leak substraction of two electrode voltage-clamp current records was carried out using as leak currents produced by hyperpolarizing pulses of the same amplitude as the test depolarizing commands. Oocytes in which leak commands elicited time-dependent currents were discarded. Averages of 10 records were used for both test and leak.

Inward currents were evoked by depolarizing, in 10 mV steps, from -60 mV to a command potential of -20 to +40 mV in 10 mV steps and from -80 mV to a command potential of -30 to +2- mV in oocytes injected with sodium channel cRNA. Current traces are blanked for the first 1.5 ms from the onset of the voltage step to delete the capacity transients for clarity. The peak current is reached at the same command voltage for the two holding potentials, but is slightly smaller from -60 mV because of steady-state inactivation.

The effects of 50% or 100% replacement of external Na+ by N-methyl-D-glucosamine on the sodium channel current were elicited by stepping the depolarizing currents given to the oocyte from -60 to +1 mV. Data were fitted with the equation h_x =

$1/(1 + \exp((V-V_{50})/k))$, where V is the prepulse potential, V_{50} the potential of 50% inactivation and the k the slope factor (best squares fit). The effect of TTX (10 μM and 100 μM) on the peak Na^+ current (test pulse from -60 to +20 mV) was also determined. The effect was quickly reversible upon washout.

After a minimum incubation of 7 days from cRNA injection, step depolarizations to potentials positive to -30mV elicited inward currents which peaked between +10 and +20 mV with an average maximum amplitude of 164 ± 72 nA (from -60 mV holding potential, n = 13) and a reversal potential of +35.5 ± 2.2 mV (n = 10). The inward current was reversed by total replacement of Na+ in the external medium with an impermeant cation (N-methyl-D-glucosamine). The current's reversal potential was shifted in 50% Na+ by 13.7 ± 3.2 mV in the hyperpolarizing direction (n = 3; predicted value for a Na+ -selective channel, 17.5 mV). The inactivation produced by a 1s prepulse was half-maximal at -30.0 ± 1.3 mV (slope factor 14.0 ± 1.7 mV, n = 5.

TTX had no effect at nanomolar concentrations, and produced only a 19.1 ± 8.3% reduction at 10 μM, n = 3). The estimated half-maximal inhibitory concentration (IC_{50}) was 59.6 ± 10.1 μM TTX.

The local anesthetic lignocaine was also weakly inhibitory, producing a maximum block of 41.7 ± 5.4% at 1 mM on the peak current elicited by depolarizing pulses from -60 mV to +10 mV (1 every min; n = 3), whereas under the same conditions 100 μM phenytoin had no effect.

A similarity with the TTX-insensitive Na+ current of DRG neurons was the effectiveness and rank order of Pb^{2+} versus Cd^{2+} in reducing peak Na^+ currents (-63.9 ± 18.1% for Pb^{2+} versus -24.4 ± 7.9% for Cd^{2+} at 50 μM and 100 μM, respectively; n = 3, P = 0.0189). The electrophysiological and pharmacological characteristics of the oocyte expressed DRG sodium channel are thus similar to the properties of the sensory neuron TTX-insensitive channel, given the constraints of expression in an oocyte system. In oocytes expressing the DRG sodium channel, the peak of the I/V plot occurred at a more depolarized potential than that of the DRG TTX-insensitive current, despite a similar reversal potential. This difference may reflect the absence of the accessory β1 subunit found in DRG, which is known to shift activation to more negative potentials when

expressed with the subunit of other Na^+ channels. In addition, splice variants that exhibit an activation threshold more negative to SNS sodium channel may shift activation to the more negative potentials observed in sensory neurons.

Example 14 - Distribution of DRG Sodium Channel in Neonatal and Adult Rat Tissuesand Cell Lines

Northern blot and reverse transcriptase-polymerase chain reaction (RT-PCR) were used to examine neonatal and adult rat tissues for expression of the DRG sodium channel messenger RNA.

Random primed ^{32}P-labeled DNA Pst -Acc1 fragment probes (50 ng, specific activity 2 x 10^9 c.p.m. per µg DNA) from interdomain region 1 (nucleotide position 1,478-1,892) of the SNS sodium channel nucleic acid sequence were used to probe total RNA extracted from tissues. The following tissues and cell lines were tested: central nervous system and non-neuronal tissues from neonatal rats; peripheral nervous tissue including neonatal Schwann cells and sympathetic neurons, as well as C6 glioma, human embryonal carcinoma line N-tera-2 and N-tera-2 neuro, rat sensory neuron-derived lines ND7 and ND8, and human neuroblastomas SMS-KCN and PC12 cells grown in the presence of NGF; adult rat tissue including pituitary, superior cervical ganglia, coeliac ganglia, trigeminal mesencephalic nucleus, vas deferens, bladder, ileum and DRG of adult animals treated with capsaicin (50 mg/kg) at birth and neonatal DRG control. Total RNA (10 µg) or 25 µg of RNA from tissues apart from superior cervical ganglion sample (10 µg) and capsaicin-treated adult rat DRG (5µg) were northern blotted.

Total RNA was separated on 1.2% agarose-formaldehyde gels, and capillary blotted onto Hibond-N filters (Amersham). The amounts of RNA on the blot were roughly equivalent, as judged by ethidium bromide staining of ribosomal RNA and by hybridization with the ubiquitously expressed L-27 ribosomal protein transcripts. Filters were prehybridized in 50% formamide, 5 x SSC containing 0.5% sodium dodecyl sulfate, 5 x Denhardts solution, 100 µg/ml boiled sonicated salmon sperm DNA (average size 300 bp),10 µg/ml poly-U and 10 µg/ml poly-C at 45°C for 6h. After 36 hours hybridization in the same conditions using 10^7 c.p.m. per ml hybridization probe, the filters were briefly washed in 2 x SSC at room temperature, then twice with 2 x SSC with 0.5% SDS at 68°C

for 15 min, followed by a 20 min wash in 0.5% SDS, 0.2 x SSC at 68°C. The filters were autoradiographed overnight or for 4 days on autoradiography film (Kodak X-omat).

For RT-PCR experiments, 10 μg total RNA from neonatal rat tissues (spleen, liver, kidney, lung, intestine, muscle, heart, superior cervical ganglia, spinal cord, brain stem, hippocampus, cerebellum, cortex and dorsal root ganglia), or 2 μg total RNA from control or capsaicin-treated rat DRG or DRG neurons in culture were treated with DNase I and extracted with acidic phenol to remove genomic DNA.

cDNA was synthesized with Superscript reverse transcriptase using oligo dT(12-18) primers and purified on Qiagen 5 tips. Polymerase chain reaction (PCR) was used to amplify cDNA (35 cycles, 94°C, 1 min; 55°C, 1 min; and 72°C, 1 min), and products separated on agarose gels before staining with ethidium bromide. L-27 primers (Ninkina et al. (1983) Nucleic Acids Res. 21, 3175-3182) were added to the PCR reaction 5 cycles after the start of the reaction with the DRG sodium channel specific primers which comprised

5'-CAGCTTCGCTCAGAAGTATCT-3' (SEQ ID NO: 9) and

5'-TTCTCGCCGTTCCACACGGAGA-3' (SEQ ID NO: 10).

Transcription of mRNA coding for the DRG sodium channel could not be detected in any non-neuronal tissues or in the central nervous system using northern blots or reverse transcription of mRNA and the polymerase chain reaction. Sympathetic neurons from the superior cervical ganglion and Schwann cell-containing sciatic nerve preparations, as well as several neuronal cell lines were also negative. However, total RNA extracts from neonatal and adult rat DRG gave a strong signal of size about 7kb on northern blots. These data suggest that the DRG sodium channel is not expressed only in early development.

RT-PCR of oligo dT-primed cDNA from various tissues using DRG sodium channel primers and L-27 ribosomal protein primer showed the presence of DRG sodium channel transcripts in DRG tissue only.

RT-PCR was also performed on DRG-sodium channel and L-27 transcripts from DRG neurons cultured and treated with capsaicin (overnight 10 μM) or dissected from neonatal animals treated with capsaicin (50 mg/kg on 2 consecutive days, followed by DRG isolation 5 days later. The signal from the L-27 probe was the same in capsaicin-treated cell cultures or animals as compared with controls that were not treated with

capsaicin. There was a significant diminution in the DRG sodium channel signal from capsaicin-treated cultures or animals as compared with controls. Control PCR reactions without reverse transcriptase treatment were also done to control for contaminating genomic DNA.

When neonatal rats were treated with capsaicin and total adult DRG RNA subsequently examined by northern blotting, the signal was substantially reduced, suggesting that the DRG sodium channel transcript is expressed selectively by capsaicin-sensitive (predominantly nociceptive) neurons. These data were confirmed by RT-PCF experiments on both cultures of DRG neurons, and in whole animal studies.

Example 15 - Distribution of DRG sodium channel in rat tissue by *in situ* hybridization

In situ hybridization was used to examine the expression of the DRG sodium channel transcripts at the single-cell level in both adult trigeninal ganglia and neonatal and adult rat DRG.

A SNS sodium channel PCR fragment of interdomain region I between positions 1,736 and 1,797 of the SNS sodium channel nucleic acid sequence was subcloned into pGem3Z (Promega, Madison, Wisconsin, USA) and digoxygenin (DIG)-UTP (Boehringer-Mannheim, Germany) labeled sense or antisense cRNA generated using SP6 or T7 polymerase, respectively. Sample preparation, hybridization and visualization of *in situ* hybridization with alkaline phosphatase conjugated anti-DIG antibodies was carried out as described in Schaeren-Wimers, et al., A. (1993) Histochemistry 100: 431-440, with the following modifications. Frozen tissue sections (10 μM-thick) of neonatal rat lumbar DRG, and adult trigeminal ganglion neurons were fixed for 10 min in phosphate buffered saline (PBS) containing 4% paraformaldehyde. Sections were acetylated in 0.1M triethanolamine, 0.25% acetic anhydride for 10 min. Prehybridization was carried out in 50% formamide, 4 x SSC, 100 μg/ml boiled and sonicated ssDNA, 50 μg/ml yeast tRNA, 2 x Denhardts solution at room temperature for 1 h. Hybridization was carried out overnight in the same buffer at 65°C. Probe concentration was 50 ng/ml. Sections were washed in 2 x SSC for 30 min at 72°C for 1 hr and twice in 0.1 SSC for 30 min at 72°C

before visualization at room temperature with anti-digoxygenin alkaline phosphatase conjugated antibodies. The same sections were then stained with mouse monoclonal antibody RT97 which is specific for neurofilaments found in large diameter neurons.

Subsets of sensory neurons from both tissues showed intense signals with a DRG sodium channel-specific probe. Combined immunohistochemistry with the large-diameter neuron-specific monoclonal antibody RT97 and the DRG sodium channel specific probe showed that most of the large diameter neurons did not express the DRG sodium channel transcript. Small diameter neurons were stained with the DRG sodium channel specific probe but not the large diameter neurons.

Example 16 - Site Directed Mutagenesis of SNS Sodium Channel - TTX Sensitivity

The SNS sodium channel is 65% homologous to the tetrodotoxin-insensitive cardiac sodium channel. A number of residues that line the channel atrium have been implicated in tetrodotoxin binding. The amino acid sequence of the SNS sodium channel exhibits sequence identity to other tetrodotoxin-sensitive sodium channels in 7 out of 9 such residues. One difference is a conservative substitution at D(905)E. A single residue (C-357) has been shown to play a critical role in tetrodotoxin binding to the sodium channel. In the SNS sodium channel, a hydrophilic serine is found at this position, whereasa other sodium channels that are sensitive to TTX have phenylalanine in this position.

Site-directed mutagenesis using standard techniques and primers having the sequence TGACGCAGGACTCCTGGGAGCGCC (SEQ ID NO: 31) was used to substitute phenylalanine for serine at position 357 in the SNS sodium channel. The mutated SNS sodium channel, when expressed in *Xenopus* oocytes produces voltage-gated currents similar in amplitude and time course to the native channel. However, sensitivity to TTX is restored to give an IC_{50} of 2.5 nM (+-0.4, n = 5), similar to other voltage-gated sodium channels that have aromatic residues at the equivalant position. The table below shows IC_{50} for SNS sodium channel, and the rat brain iia, muscle type 1, and cardiac tetrodotoxin-insensitive sodium channels.

TTX Sensitivity

Sodium Channel	ss1 domain	ss2 domain	IC_{50}
Rat brain iia	FRLM	TQDFWENLY	18 nM
muscle type 1	FRLM	TQDYWENLY	40 nM
cardiac TTXi	FRLM	TQDCWERLY	950 nM
SNS	FRLM	TQDSWERLY	60 micromolar
SNS mutant	FRLM	TQDFWERLY	2.5 nM

FRLM - SEQ ID NO: 11; TQDFWENLY - SEQ ID NO: 12;
TQDYWENLY - SEQ ID NO: 13; TQDCWERLY - SEQ ID NO:14;
TQDSWERLY - SEQ ID NO: 15; TQDFWERLY - SEQ ID NO:16

Example 18

Polyclonal antibodies were raised in rabbits against the following peptides derived from the SNS sodium channel protein amino acid sequence:

Peptide 1 TQDSWER (SEQ ID NO:17)
Peptide 2 GSTDDNRSPQSDPYN (SEQ ID NO: 18)
Peptide 3 SPKENHGDFI (SEQ ID NO: 19)
Peptide 4 PNHNGSRGN (SEQ ID NO: 20)

The peptides were conjugated to Keyhole limpet heocyanin (KLH) and injected repeatedly into rabbits. Sera from the rabbits was treated by Western blotting. Several sera showed positive results indicating the presence of antibodies specific for the peptide in the sera.

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